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BIOLOGICAL BULLETIN

THE MITOCHONDRIA AND OTHER STRUCTURES OBSERVED BY THE TISSUE CULTURE METHOD IN THE MALE GERM CELLS OF *CHORTHIPPUS CUR- TIPENNIS* SCUDD.¹

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INTRODUCTION.

A study of the chromosomes of *Chorthippus curtipennis* by Robertson (in press) led to the desire to study the mitochondria and other structures of the cytoplasm in order to determine if possible the bearing of the cytoplasmic structures upon the later development.

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It was observed that this material lent itself to the study of the living cell by means of the tissue culture method as described for the chick embryo cell by Lewis, M. R., and Lewis, W. H. ('15) and that not only could the most minute structures of the cell be observed from day to day, but also these structures could be experimented upon as readily as those of the chick embryo. It was decided to study the mitochondria and other cytoplasmic structures of the germ cells of *Chorthippus curtipennis* by means of the tissue culture method.

LITERATURE.

Living Material.—The earliest observations upon the living germ cells of the Arthropods were those of von La Valette St. George ('86) in which he made a careful study of the Nebenkern of the spermatid and described the structure and behavior of that body more completely than many of the later investigators.

Chambers, R. ('15), in his microdissection studies on the germ cell, for which he used the male germ cells of *Disosteira carolina* (grasshopper) and of *Periplaneta americana* (cockroach), gives many interesting observations as to the behavior of the mitochondria during the spermatocyte divisions and he also describes in detail the development of the axial filament of the spermatid and spermatozoön, but apparently Chambers made no effort to trace the cytoplasmic structures of the germ cells throughout their development. The description of the mitochondria during the spermatocyte divisions and the formation of the Nebenkern of the spermatid agrees in general with that found for preparations of *Chorthippus curtipennis* when stained with Janus green. The development of the spermatozoön of *Chorthippus*, however, takes place in quite a different manner from that described by Chambers for the cockroach.

Goldschmidt, R. ('15) states that it was possible to keep the sperm cells of the moth *Samia cecropia* L. alive for three weeks in cultures of hæmolymph and that during this time many follicles finished the process of spermatogenesis. Goldschmidt does not describe the process of spermatogenesis, but merely states that it corresponds with that described for fixed preparations. From these studies upon *Chorthippus* it is quite evident that neither the

details of the cellular structure nor their behavior could be carefully studied while the cells remained in the follicle as described by Goldschmidt, but that for this purpose it is necessary to observe isolated cells which lie close to the cover slip.

Fixed Preparation.—The mitochondria and other structures present in various types of fixed material have been studied more or less in detail by numerous observers, with results which depend very largely upon the state of preservation of the material studied. Duesberg, J. ('11) has reviewed this literature so it is unnecessary to repeat it here. In an earlier paper Duesberg, J. ('10) gives a clear and complete description of the behavior of the mitochondria in *Blatta germanica*, which corresponds in all but a few details with that given below for *Chorthippus curtipennis*.

METHOD.

The cultures were prepared in the usual manner (Lewis, M. R., and Lewis, W. H., '15) and all precautions were observed in order to keep them not only chemically clean but also aseptic, except in cases where the period of observation was to extend over only a short time, as for instance when a vital stain was used.

Various different culture media were tried and the one which appeared to be most nearly isotonic with the body fluid of the grasshopper and which also was most favorable for growth was practically Locke's solution *i. e.*, NaCl 0.9 per cent., CaCl_2 0.025, KCl 0.042 per cent., NaHCO_3 0.02 per cent., dextrose 0.25 per cent., peptone 0.2 per cent., but since the observations were made at Woods Hole where running sea water is supplied, the same concentration of salts was obtained by a dilution of the sea water as follows: sea water 30 c.c.+distilled water 50 c.c.+bouillon 20 c.c.+dextrose 0.25 gram+ NaHCO_3 0.02 gram. The bouillon was prepared in the same manner as that used for bacteriology, except in this case grasshopper muscle was used in place of beef. A solution of peptone alone can be substituted for the bouillon with rather good results. The culture medium, which is successful, depends largely upon the amount of evaporation which takes place in the technic of the individual observer. This can be determined from the appearance of the preparation itself, for it was found that when the medium was too concen-

trated the cells formed numerous delicate pseudopodia or flagella and when the medium was too dilute the cells became swollen. The solution which is most nearly isotonic is one in which the cells remain round and flatten out close to the cover slip, or crawl along the cover slip by means of broad, flat pseudopodia.

The grasshopper was opened on the ventral side by means of sterile scissors and the walls pinned down with sterile pins. The testis follicles were then removed aseptically. Each follicle of the testis is made up of a number of cysts, each of which contains a number of cells all in the same stage of development. The apical cell and the primary spermatogonia are at the blind end of the follicle. The cysts which contain the secondary spermatogonia, the first spermatocytes, the synapsis stages, the growth stages, the first spermatocyte division stages, the second spermatocytes, the second spermatocyte divisions, the spermatids, and the spermatozoa are arranged in order back of this towards the open end of the follicle. In order to obtain the cells in the stage desired for observation, a follicle of the testis was placed in a thin drop of the sterile culture medium on a sterile cover slip and, with the aid of a binocular microscope, the wall of the cyst, which contained the cells to be studied, was punctured with a sharp, sterile needle so that the cells of the cyst flowed out into the medium. The excess of the medium was drawn off by means of a capillary pipette and the preparation was then sealed onto a hollow ground slide by means of a vaseline ring. In case stained preparations were to be observed, the vital stain, Janus green or neutral red, was dissolved in the drop of the culture medium in which the follicle was punctured. The cells released from the cyst wall spread out in a thin layer along the cover slip and were then studied by means of the No. 6 ocular and 2 mm. oil immersion lens. A 40-watt Mazda electric light was used for illumination.

Since any stage in the development of the germ cell can be obtained in the above manner, it was not found necessary to watch any one cell over a long period of time, although the cultures remained healthy and dividing cells were found as late as the fourth day.

Tissue cultures of the germ cells in body fluid medium were

made as controls, but they were not so useful for the study of the cell structure owing to the fact that the medium is more opaque and that the cells do not spread out in a thin layer close to the cover slip. Also the plasma medium is more difficult to use for experimental purposes owing to the fact that it is easily coagulated.

OBSERVATIONS.

General.—When the cyst wall is broken the cells flow out and become attached to the cover slip. The cells appear to be formed of a clear homogeneous cytoplasm which contains a nucleus and granules. Numerous cells, which contain two or four nuclei and also a correspondingly increased amount of mitochondria, were observed in all stages of development as, for instance, a first spermatocyte with two nuclei and a double amount of mitochondria or a young spermatid with two or four nuclei and also two or four nebenkern. During observations upon one unstained preparation two second spermatocytes whose cytoplasm touched at one point were observed to fuse into a single cell (Figs. 27, 28, 29). The fused cell, which resulted from the two single cells, contained two groups of chromosomes and two groups of mitochondria. In certain stages in the development of the germ cell the granules are scattered throughout the greater part of the cytoplasm (spermatogonium), while in other stages the granules are limited to a definite area (division of spermatocytes). There is no indication of any network structure either of the cytoplasm or of the nucleus. The cells of a cyst remain attached to each other by a long thread-like process, or in some cases by a short thick process, which appears as though the cells had not been completely separated at division, but had remained attached by a band of cytoplasm. Groups of spermatozoa are attached by one end to a crescent-shaped body, while the other end is free and lashes about continuously. Several of these crescent-shaped bodies, each with numerous spermatozoa attached to it, may be seen in one field. The cells may send out broad, flat pseudopodia and crawl along the cover slip, or in media which are too concentrated or to which Janus green has been added, the cells may send out numerous delicate pseudopodia, which appear more like flagella. However, other factors

besides the concentration of the medium may influence the size of the pseudopodia, for Goldschmidt (1915) states that the germ cells form flagella in the cultures in hæmolymph and that these flagella can be caused to appear and disappear by a change of temperature.

During mitosis the mitochondria become long, delicate threads and lie around the spindle in such a way as to be easily mistaken for the spindle (Figs. 14 and 22). In none of our preparations were the spindle threads seen and the spindle itself did not show as a cone of material, which had a different light refraction, as it did in the chick. In one preparation the position of the spindle at one pole was outlined (Fig. 16), but even in this case no spindle threads were seen.

The spindle is present however and can be readily shown by means of acetic acid vapor, which destroys the mitochondria and coagulates the cytoplasm sufficiently to show the spindle practically the same as it is shown in figures drawn from fixed material (Figs. 21-26).

Vital Stains.—All stages in the development of the germ cell were studied, not only by means of the living unstained cell, but also by means of preparations stained with Janus green and others stained with neutral red. A few preparations were stained with both Janus green and neutral red. The Janus green stain and also the neutral red stain were dissolved in the culture medium in exceedingly dilute solutions, never more than 1-50,000 parts and frequently as dilute as 1-100,000 parts.

The neutral red stains a large round granule, which is quite different from the mitochondria, not only in size and appearance, but also in behavior (Figs. 1, 22, 36, 37, 39, 41, 46 and 49). This granule has the same reaction to Brilliant cresylblue 2 b. as that of the granule described by Lewis and Lewis ('15) in connection with the "vacuole" and agrees in a few details with the "beta globule" described by Coghill ('15). In a few cases the granule reacts with the neutral red stain in the same manner as does the neutral red granule, which Renaut, J. ('04) and Dubreuil, G. ('13) describe in connection with the connective tissue development. Owing to the fact that the literature does not furnish a satisfactory term for this granule, it will be called simply the neutral red granule in the following observations. Duesberg ('10) in certain

figures, as for instance those of the spermatid, shows a granule in the same position as that of the neutral red granule in our preparations, and, although the granule is stained like the mitochondria with Benda's stain, Duesberg states that it is in all probability not mitochondria. In preparations stained with Janus green this granule remains unstained. The somatic cells, which form the wall of the follicle and also the apical cell (Figs. 1 and 2), are full of these bright red granules when the preparation is stained with neutral red, but the germ cells contain only a very few neutral red granules.

With Janus green stain however, the germ cells are shown to contain abundant mitochondria in the form of granular threads or of small rod shaped granules. After the preparation has been stained for a short time the granules coalesce into larger globules (Figs. 4 and 5) and finally they disappear in the cytoplasm. Long, thread-like mitochondria rapidly break into granules when stained with Janus green (Figs. 13-20).

Neither of the above stains showed the spindle threads during mitosis. There was no appearance seen at any time during observation upon either the unstained cell or the cell stained by means of the above vital stains, which could lead one to conclude that the mitochondria are formed from any material at the expense of the nucleus as Wassilieff ('07) and his followers contend.

Nucleus.—The various changes which the nucleus undergoes during the so-called resting stage and during division can be clearly observed throughout the development of the germ cells from spermatogonia to spermatozoa. In the resting nucleus of any stage chromatin threads were observed. In the nucleus of the spermatogonium these chromatin threads or spiremes seem to fill the nuclear space like so many sacs (Fig. 1). When the preparation was stained with neutral red, the walls of these sacs became faintly pink and so revealed the boundaries of the chromosomes. During the telophase, and in a few cases, in the late anaphase of the spermatogonium, the spermatocyte and the spermatid, the chromosomes have a granular structure (Figs. 4, 10, 20). These granules appear to be uniform in size and it might prove possible to count the number of granules which compose a given chromosome. The importance of these granules in "crossing over" phenomena may appear later.

The number and also the characteristics of the chromosomes observed in the living cells correspond with what was found in the fixed preparations. In the first spermatocyte the chromosomes were as follows: Five "short rod" tetrads, three large "compound ring" tetrads, derived from the three pair of compound V chromosomes of the spermatogonium and the rod-like sex chromosomes. In this genus, *Chorthippus* formerly known as *stenobothrus*, there is a peculiar compounding of six pairs of rod chromosomes to form the three pairs of V's that are characteristic so far as is known of all the species of the genus (Meek, '11, '12; Gerard, '09; Davis, '08).

This peculiar process by which the three pairs of compound V chromosomes are formed was first observed by Robertson (in press). The subsequent behavior of these compound chromosomes was the same in the living cell as has been described from the fixed preparations and the five rods, three V's and the sex chromosomes (present only in one of the two daughter cells, which result from the first spermatocyte division) were easily identified in the second spermatocytes and in the spermatids.

The Apical Cell.—The apical cell lies in the blind tip of the follicle surrounded by primary spermatogonial cells (Figs. 1, 3). It is a round cell, which contains a more or less oval nucleus, and is attached to the walls of the follicle by several thick cell processes. The apical cell contains both mitochondria and neutral red granules (Figs. 1 and 3). The former are fewer in number than the latter and uniformly small in size (Fig. 3). They are arranged as granular threads mostly in a layer around the nucleus. The neutral red granules are considerably larger than the mitochondria and are scattered throughout the cytoplasm and in the cell processes. When a preparation is stained with neutral red, these granules in the apical cell and also in all of its processes rapidly take up the stain, so that the apical cell becomes quite red in appearance. While the few granules in the spermatogonia which surround the apical cell take up the stain only after a long time and then only in a few scattered granules so that the spermatogonia appear practically colorless. The somatic cells (Fig. 1), which form the wall of the follicle, have abundant neutral red granules and these stain with neutral red in much the same

manner as did those of the apical cell. This striking resemblance of the apical cell to the somatic cells in contrast to the germ cells suggests the possibility that the apical cell may be more closely related to the somatic cells than to the germ cells.

Primary Spermatogonia.—In the primray spermatogonia both the mitochondria and the neutral red granules can be identified in the unstained cell. In the resting cell the mitochondria appear as delicate granular threads and at this time these threads seem to radiate from the distal pole of the cell (*i. e.*, the region of the last connection with its sister cell at mitosis). The mitochondria of the primary spermatogonia stain less intensely with Janus green than does that of cells in a later stage of development and when stained with Janus green the delicate threads become rapidly distorted and appear as granules. The neutral red granules, from 4 to 10 or 12 in number, are larger, more or less round and much more refractive than the mitochondria granules. These neutral red granules, so far as was seen, did not seem to be located in any definite region of the cell, but were scattered through the cytoplasm.

Secondary Spermatogonia (Period of Multiplication).—The secondary spermatogonial cells are smaller than the primary spermatogonia and the mitochondria are usually in the form of fine, granular threads scattered from the distal end of the cell towards the nucleus. As the cell approaches the resting condition the mitochondria become more uniformly scattered throughout the cytoplasm. In a few observations the mitochondria appeared to be absent from a region at the extreme distal pole of the cell, possibly the mitosome (*i. e.*, the remains of the spindle, Figs. 2, 4, 6).

During mitosis the mitochondria arrange themselves as long threads close to the spindle and frequently they have the appearance of abnormally thick spindle threads. During the constriction of the cytoplasm at late anaphase the mitochondria threads again become granular and at telophase the mitochondria are separated into two practically equal amounts, one of which passes into each daughter cell and from this mass the mitochondria migrate towards and partly around the nucleus (Fig. 4), as can be seen in the first spermatocyte.

First Spermatocyte (Growth Period).—As the cell grows in size the amount of mitochondria appears to increase correspondingly and certainly the mitochondria of the spermatocyte stain much more intensely with Janus green than do those of the spermatogonium. The neutral red granules are few in number and scattered throughout the cytoplasm.

In the later growth period the mitochondria become grouped into two, or in a few cases, possibly more masses (Figs. 7 and 8 and 11). Unfortunately the significance of this was not comprehended, but it is without doubt closely allied with some change in the cell itself, as the massed arrangement of the mitochondria is quite characteristic of the synapsis stages. There was no evidence that the mitochondria granules paired during synapsis, although the pairing of the chromosomes was clearly seen.

First Spermatocyte Division.—During the prophase and early metaphase of the first spermatocyte, the mitochondria migrate away from the two masses of mitochondria granules and elongate towards the two poles of the spindle (Fig. 12), so that when the chromosomes are arranged on the spindle plate, the mitochondria appear as threads which more or less closely surround the spindle. As the chromosomes move apart, the mitochondria become drawn out into straight, even threads closely attached to the spindle between the two groups of chromosomes (Figs. 13, 14 and 15). Since they are much longer and more refractive at this stage, they may be easily mistaken for the spindle fibers and some cytologists have stated that the mitochondria in the germ cell are but the remains of the spindle fibers. A simple experiment shows that this is not the case in *Chorthippus curtipennis*. Figs. 21, 22 and 23 were made from the living cells in the cultures and then an opening was made in the vaseline ring, which supported the cover slip and a drop of glacial acetic acid was placed on the slide near to the opening so that the fumes from the acid passed through the opening and acted upon the preparation. The mitochondria were destroyed at once and became lost within the coagulum of the cytoplasm, and, where previously no spindle could be seen, there now appears a typical spindle (Figs. 24 and 25). Fig. 26 shows the remains of the spindle fibers near the periphery of each daughter cell, but the mitochondria,

which previously lay along these threads (Fig. 23) and scattered towards the nucleus, have now disappeared.

During the anaphase of the living cell the daughter chromosomes move to the extreme proximal pole of the cell and when the cytoplasm constricts, the continuous mitochondria threads again become granular and from the ends of these threads the mitochondria granules migrate away towards the two groups of chromosomes (Fig. 22). In some instances the entire mitochondria thread appeared to pass over to one daughter cell, but usually the division of the mitochondria took place by means of the migration of the granules into each daughter cell, so that each daughter cell received about the same amount of mitochondria.

When the preparation was stained with Janus green the mitotic division continues, provided it was started before the stain acted upon the cell (Figs. 17-20), but after the division was finished, the cell did not pass through the second spermatocyte division as the unstained cells did. The mitochondria threads become granular and do not usually extend from chromosome group to chromosome group, but more frequently lie in a broad band around the equator of the spindle (Figs. 16, 17, 18 and 19). The ends of the granular threads swell up and become varicose before the granules migrate away (Figs. 19 and 20).

Second Spermatocyte (Interkinesis).—After the mitochondria have migrated into the daughter cells the cytoplasmic bridge between the two cells remains and elongates. The cells may sometimes remain thus attached by a long process during the semi-resting condition (interkinesis), which may continue for an hour or even for several hours before the cells prepare for the second spermatocyte division. The second spermatocyte has a characteristic appearance and is easily recognized. The chromosomes remain and it can be seen that they are no longer tetrads as they were in the first spermatocyte (Fig. 30). The mitochondria are gathered into an irregular body at one side of the cell and from this body the mitochondria granules spread out somewhat toward the nucleus (Fig. 30).

Second Spermatocyte Division.—The behavior of the mitochondria during the second spermatocyte division is practically the same as that during the first spermatocyte division. In this

case however there is usually only one mitochondrial mass (although it may divide into two in some cases) and this mass lies at one side midway between the two poles of the spindle (Fig. 30). The mitochondria migrate away from this granular mass and elongate towards the two poles of the spindle. Usually the elongated threads spread out around the spindle, but in a few cases they have been seen to remain mostly on the side of the spindle where the mitochondrial body was. The threads become homogeneous and appear to be continuous threads stretched between the two groups of chromosomes. When the cytoplasm constricts during anaphase the granules migrate into the daughter cells, but they do not usually scatter around the nucleus. A few neutral red granules are present in the cytoplasm throughout the division and can be seen in the cytoplasm on each side of the mitochondrial body in the spermatid (Figs. 36 and 37). When division is completed the mitochondria granules become contracted into a compact, spherical granular body, the *nebenkern*, and a few neutral red granules are irregularly scattered on each side of the *nebenkern*. It is interesting to note that after acetic acid vapor, a round body can still be distinguishable in the place of the *nebenkern*, so that either the mitochondria are at this stage more resistant to acetic acid, or else there is some other body present in the *nebenkern*.

In a few cases the daughter cells seemed to be unequal in size. It has not been determined whether the inequality in size of the daughter cells is due to the presence of the sex chromosome in the larger cell. Davis ('08) and Gerard ('09) in their figures for this genus each show one of the daughter cells, which result from the second spermatocyte division, larger than the other, but they do not call attention to this point.

The Spermatid and Spermatozoön.—The development of the spermatid into the spermatozoön is by no means a simple process and, even after prolonged observation, the significance of the various steps is not clear (Figs. 38–51).

The neutral red granules do not play an active part, but remain about the same in the cytoplasm until with the growth of the tail the cytoplasm becomes small in quantity and they disappear. They were not observed in the more adult spermatozoön except

in cases where some abnormal factor caused the cytoplasm along the tail of the spermatozoön to become clumped into nodes, and, in this case, one or more neutral red granules were present in each node (Fig. 49). In the young spermatid the mitochondria are in the form of the granular nebenkern (Fig. 38), which later becomes a clear, homogeneous, spherical body close to the nucleus. No trace of granules can be seen either in the unstained cell or in those stained with Janus green. Within a short period of time ($\frac{1}{2}$ hour) certain delicate threads appear within the clear nebenkern (Figs. 39 and 40). These threads may be concentric or otherwise coiled and may represent either the rows of granules under pressure, or they may be the edges of the membrane, which separates the nebenkern into two half spheres, seen at different levels, for very shortly after the threads appear, the nebenkern slides apart into two half spheres and at once becomes granular again (Figs. 41, 42, 43).

The behavior of the centrosome and the formation of the axial filament, which has been described from fixed material, was seen only in one case, and in that case it was not possible to ascertain just what connection there is between the division of the nebenkern and the formation of the axial filament. The centrosome was seen as a clear paired body near the nucleus, but at a distance from the mitochondrial body (Fig. 38). Later the centrosome occupies a position at the posterior pole of the nucleus near the Nebenkern. After the division of the Nebenkern it was seen as a small opaque body close to the nucleus (Fig. 44). The body is double and, although small, it increases in size with the growth of the tail and later becomes the middle piece (Figs. 46, 49, 50 and 51). From this body the axial filament probably is given off and later grows out into an extension of the cytoplasm.

The mitochondrial bodies, or the two half spheres of the nebenkern, now elongate as granular sacs (Figs. 45 and 46). The wider end is towards the nucleus and the sacs gradually taper off until they become only a few granules at the other end (Fig. 46). As the tail grows out these bodies grow out one on each side of the axial filament. The sacs do not increase in size, but simply spread out along the axial filament and become crowded into the narrow layer of cytoplasm along the tail, so that as the tail

elongates the granules form two irregular granular strands, which later fuse into two continuous threads of even width extending from the centrosome body or middle piece to almost the end of the tail (Figs. 49, 50 and 51).

Duesberg ('10) describes a small body composed of mitochondria at the tip of the head, but in this form such a body was not seen either in the living spermatozoön or in those stained with Janus green.

Unfortunately it was impossible to study this material in all its details during the short time at Woods Hole and there are many interesting points to be followed out, which it is hoped may be continued in later studies upon another species of grasshopper by Robertson.

DISCUSSION.

The above observations show that there are present in the male germ cell from its earliest appearance throughout its development certain granules which correspond to the mitochondria of somatic cells. The mitochondria behave in a definite and characteristic manner during the development of the male germ cell and assume a shape and position characteristic for each stage in the development. The mitochondria of the germ cell become slightly blackened with osmic acid, are destroyed by acetic acid and stained by Janus green and the usual stains for mitochondria in fixed material. That they are not the remains of spindle fibers has been clearly shown by means of acetic acid, which destroys the mitochondria and causes the spindle fibers to appear. On the other hand it does not seem possible that bodies, which have to do only with the metabolic activities of the cell should necessarily undergo such an exact behavior as is shown for instance by the division of the *nebenkern* into equal parts and the development of these two sacs of mitochondria into the two long threads of mitochondria in the spermatozoön. Neither does the behavior of the mitochondria seem to be entirely dependent on changes within the cell, as for instance during the division of the cell or the growth of the tail, where it might appear that the elongation, and at the same time narrowing of the tail, might force the mitochondria granules to assume the position of two continuous threads, for in some cases where the tail does not develop

as normally but the axial filament grows out within the cell, the mitochondria develop as normally in connection with the axial filament and form the same long threads, although in this case these threads are wound round and round within the cell (Figs. 47 and 48).

The origin of the mitochondria is still unsolved. They certainly do not arise in the male germ cells, since they are already present in the earliest germ cell. There is no evidence in the above observations that the mitochondria are formed at the expense of any nuclear material. Also there is no evidence that the mitochondria of the male germ cell of *Chorthippus curtipennis* can have any influence upon inheritance, as was suggested by Meves ('13) in his work on *Ascaris*, unless it can be shown that the tail as well as the nucleus of the spermatozoön enters the egg.

CONCLUSION.

The mitochondria as well as the neutral red granules are present in the primary spermatogonium of *Chorthippus curtipennis* and, while the neutral red granules appear to have no definite behavior, the mitochondria do behave in a characteristic manner throughout the development of the male germ cells. By means of the tissue culture method the mitochondria can be seen to be present as small, delicate granules in the primary spermatogonium. They increase in amount during the growth stage and arrange themselves along the spindle in a definite manner during the spermatocyte division. They form the nebenkern of the spermatid and from this develop into two equal homogeneous threads in the tail of the spermatozoön.

WOODS HOLE, MASS.,
September, 1915.

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EXPLANATION OF PLATES.

J. G. = Janus green stain.

N. R. = neutral red stain.

Mitochondria = black-inked granules and threads.

Neutral red granules = gray granules outlined with black ink.

Centrosome = clear round granule about same size as neutral red granule.

PLATE I.

FIG. 1. N. R. Blind end of follicle. Apical cell surrounded by primary spermatogonia. The somatic cells form the boundary of the follicle. Round neutral red granules in the apical cell and also in the somatic cells. Leitz Binoc. $4b \times 1/12$ lens.

FIG. 2. Unstained cell. Primary spermatogonium. Mitochondria are granular threads. Clear region at upper side of the cell is probably the mitosome (spindle remains). Leitz Binoc. $4b \times 1/12$ lens.

FIG. 3. J. G. and N. R. Apical cell. Mitochondria are small black granules and the neutral red are the larger gray granules. Zeiss 6 oc. 2 mm. lens.

FIG. 4. J. G. Two secondary spermatogonia still attached together. Chromosomes granular. Telophase. Mitochondria are not in nucleus but around it. Leitz Binoc. $4b \times 1/12$ lens.

FIG. 5. Same cell as Fig. 4. Globules of blue which form after the mitochondria have lost their stain.

FIG. 6. J. G. Secondary spermatogonium.

FIG. 7. J. G. First spermatocyte, centrosome divided. Sex chromosome. Early prophase.

FIG. 8. J. G. First spermatocyte. Diplotene nucleus stage. Centrosomes. Two masses of mitochondria, one on upper surface of cell and one on the lower. Leitz Binoc. $4b \times 1/12$ lens.

FIG. 9. Unstained. First spermatocyte 'bouquet-stage' synapsis. Zeiss 6 oc. 2 mm. lens.

FIG. 10. J. G. Spermatogonium, telophase. Each chromosome is in a separate vesicle and is granular. Nuclear wall reforming from chromosome vesicles. Leitz Binoc. $4b \times 1/12$ lens.

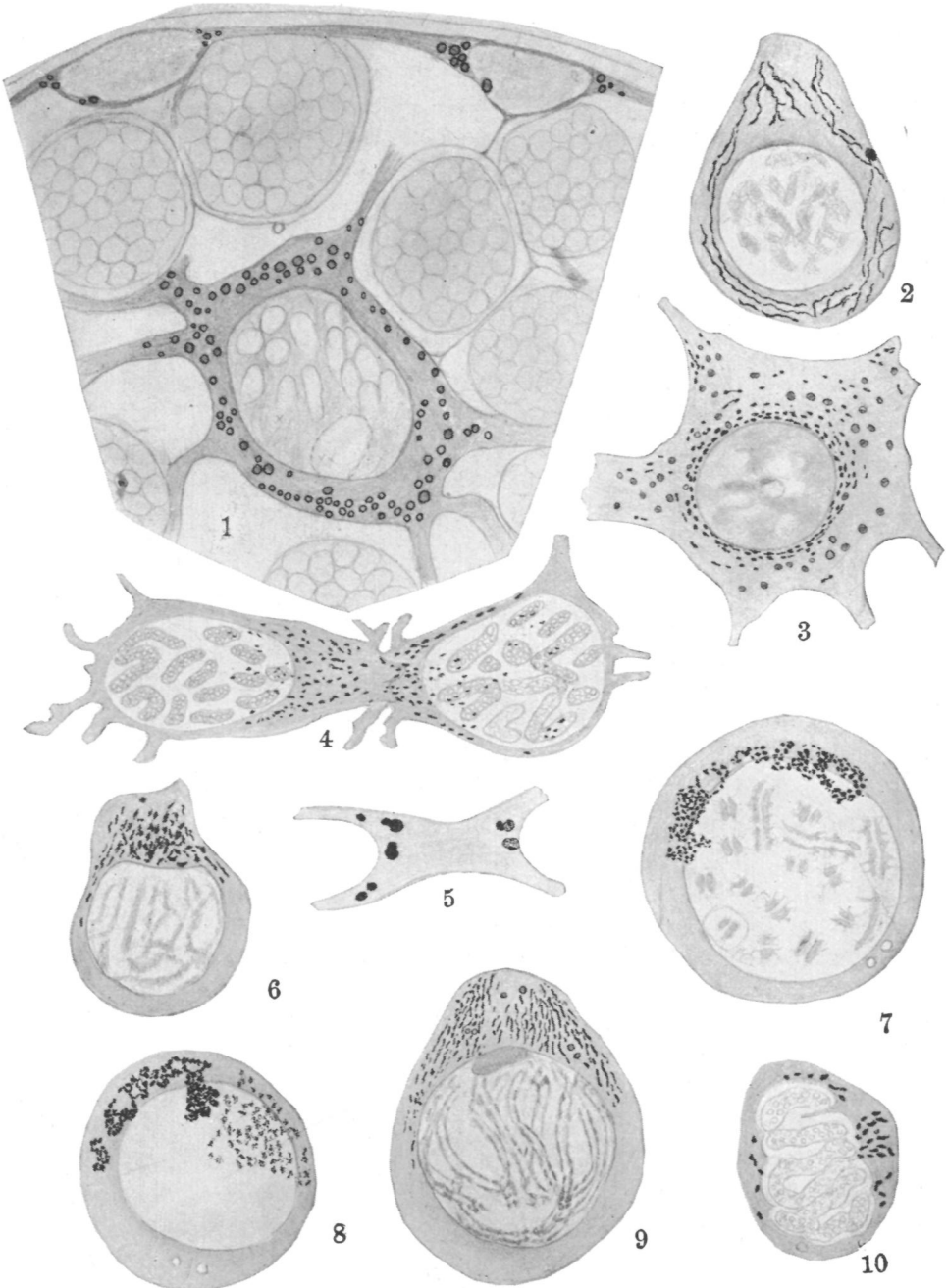


PLATE II.

FIG. 11. J. G. First spermatocyte, late prophase. Nuclear wall still intact, two masses of mitochondria.

FIG. 12. Unstained. First spermatocyte metaphase. Mitochondria threads have migrated towards the poles of the spindle. Zeiss 8 oc. 2 mm. lens.

FIGS. 13, 14, 15. Unstained. First spermatocyte division. Same cell at 9:30 A.M. (Fig. 13), 9:45 A.M. (Fig. 14), and 10:10 A.M. (Fig. 15). Mitochondria are long threads close to the spindle fibers. Zeiss 4 oc. 2 mm. lens.

FIG. 16. Unstained. Mitochondria are granular threads around the spindle. Those in the middle of the spindle occur underneath. The sex chromosome at one pole. Spindle outlined at one pole. Leitz Binoc. 4b $\times 1/12$ lens.

FIGS. 17, 18, 19. J. G. First spermatocyte division in the same cell. Fig. 17 at 9:40 A.M., Fig. 18 at 9:50 A.M., and Fig. 19 at 10:45 A.M. Zeiss 6 oc. 2 mm. lens.

FIG. 20. J. G. End of first spermatocyte division. Mitochondria migrated into two daughter cells. Chromosomes granular. Leitz Binoc. 4b $\times 1/12$ lens.

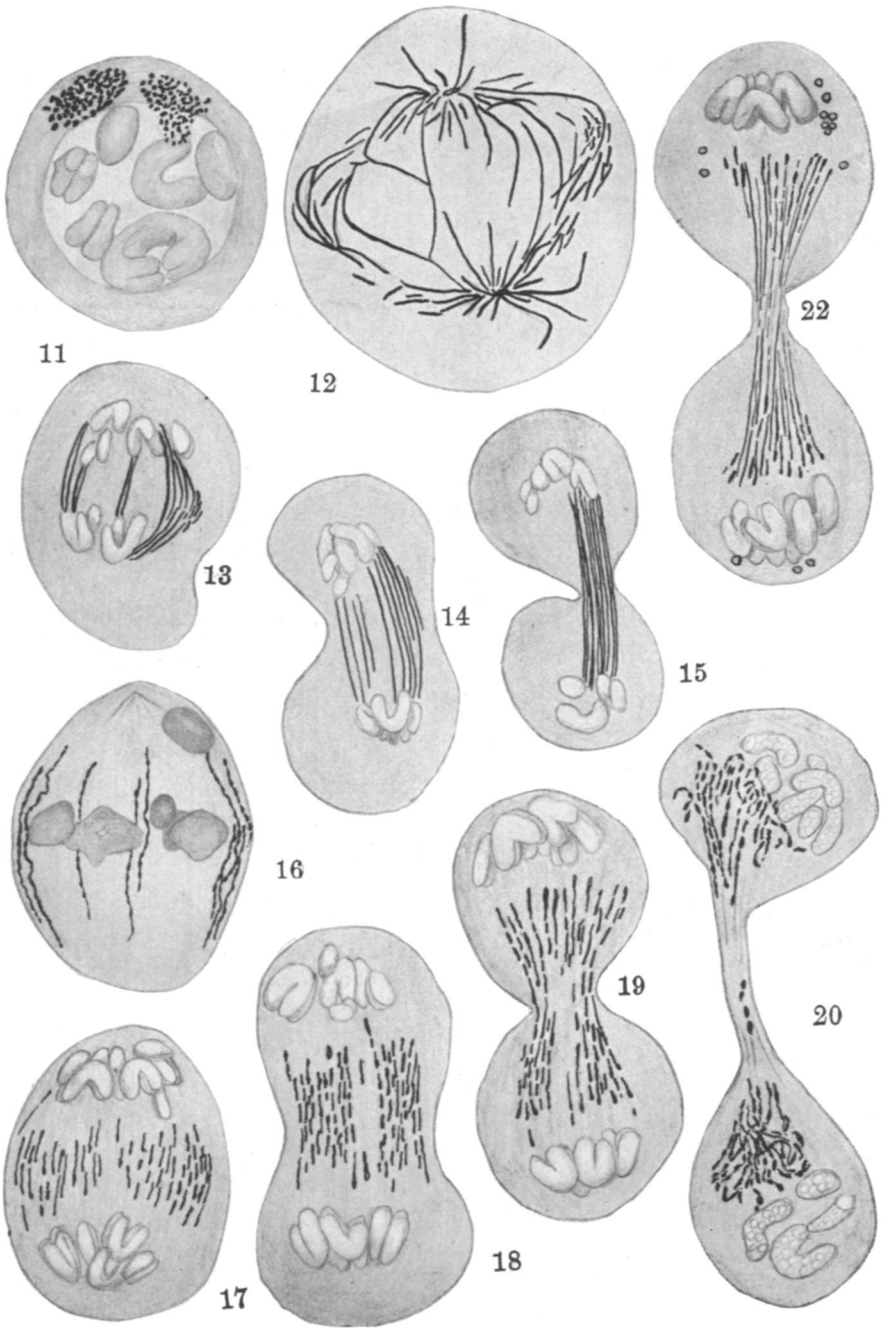


PLATE III.

FIGS. 21, 22, 23. Unstained. Division stages drawn to show mitochondria before the action of acetic acid. Zeiss 8 oc. 2 mm. lens.

FIGS. 24, 25, 26. Same cells after acetic acid fumes. Mitochondria have disappeared and the cytoplasm is coagulated. Spindle fibers can now be seen.

FIGS. 27, 28, 29. Unstained. The fusion of two cells to form a single double cell with two groups of chromosomes and two masses of mitochondria. Leitz Binoc. 4b $\times 1/12$ lens. Chromosomes not all shown.

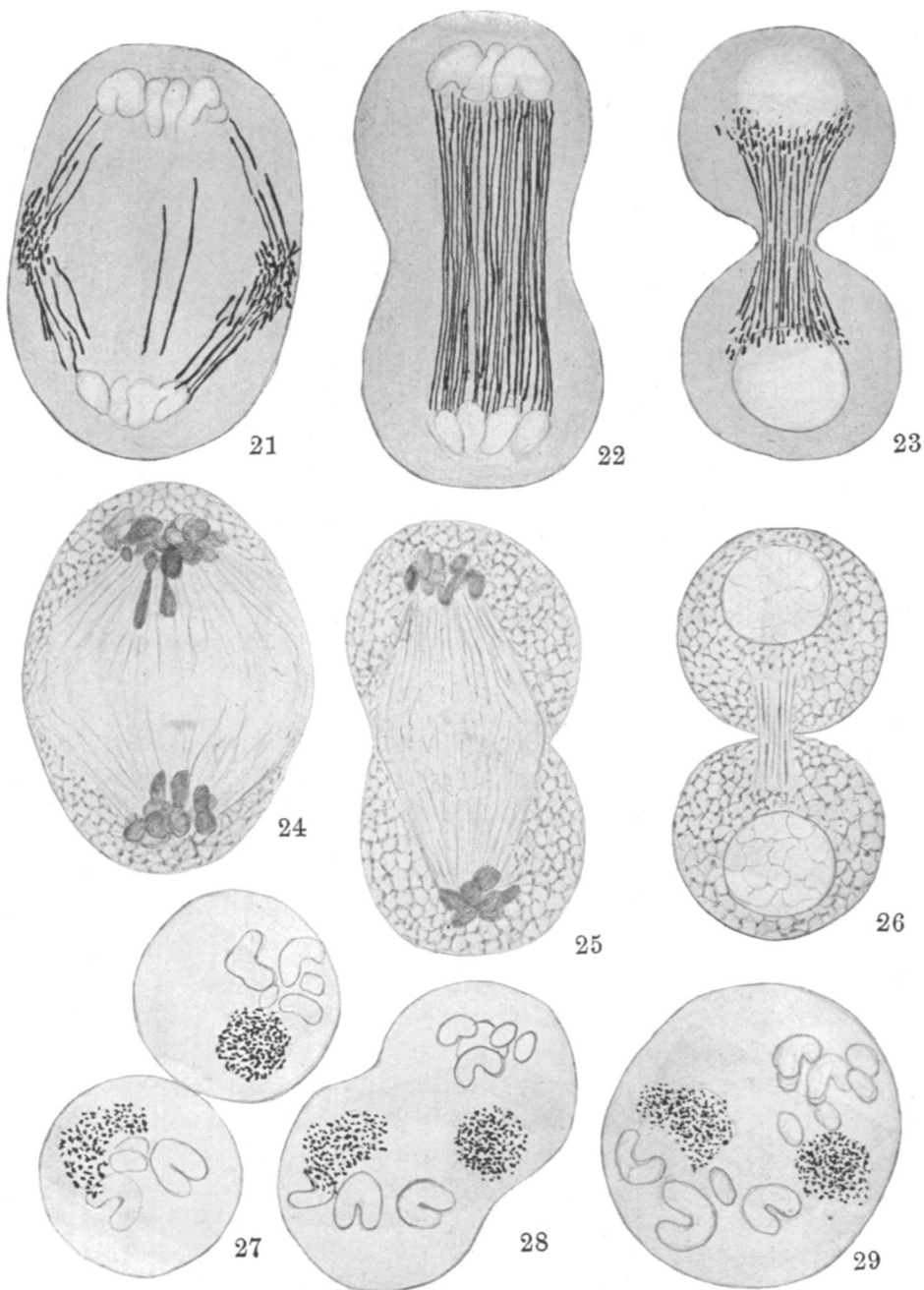


PLATE IV.

FIG. 30. Unstained. Second spermatocyte. Single mass of mitochondria from which granules are elongating toward each pole of the spindle. Zeiss 6 oc. 2 mm. lens.

FIGS. 31-35. Unstained. Different stages of a cell in the second spermatocyte division. Fig. 31 at 10:40 A.M.; Fig. 32 at 11:20 A.M.; Fig. 33 at 12:30 P.M.; Fig. 34 at 1 P.M.; and Fig. 35 at 2 P.M. The mitochondria form the nebenkern.

FIGS. 36, 37. N. R. Neutral red granules present during the second spermatocyte division. Zeiss 6 oc. 2 mm. lens.

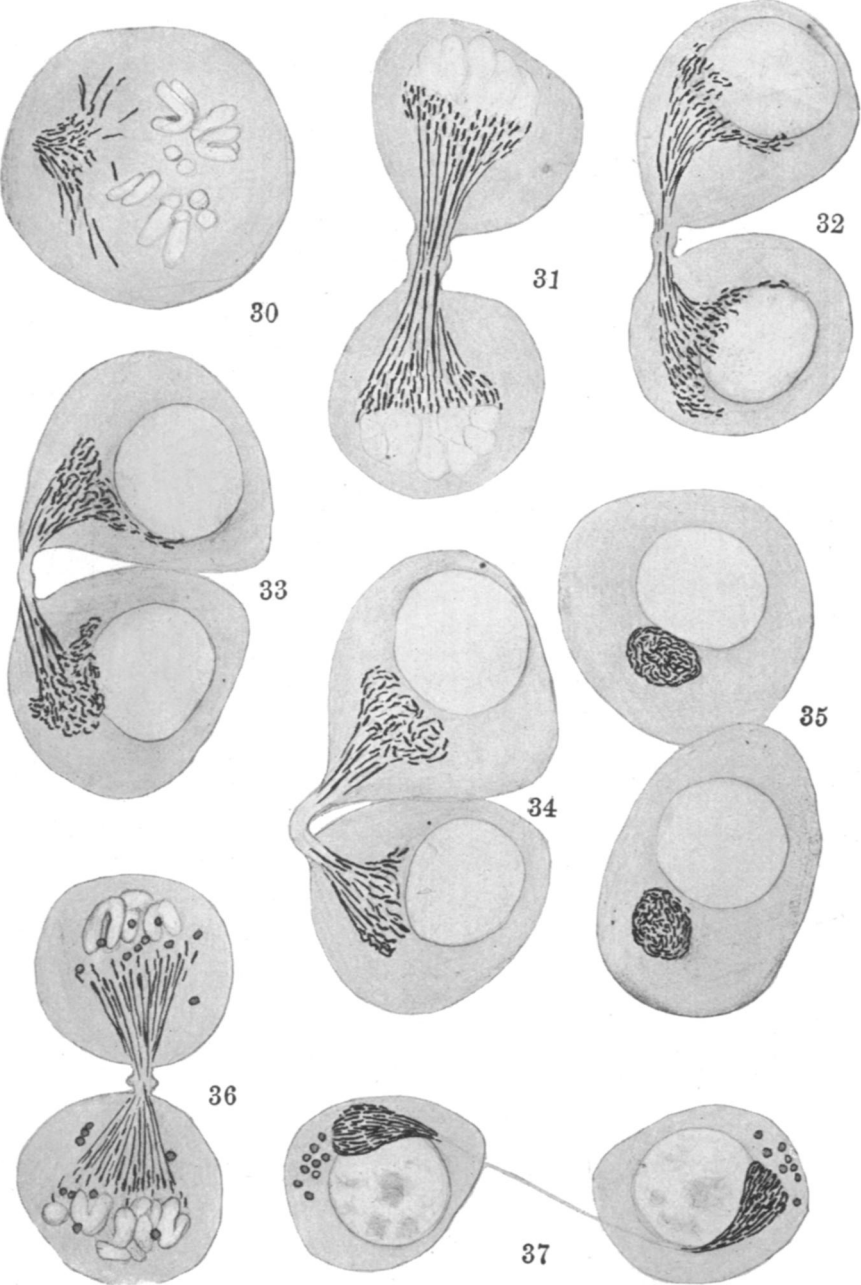


PLATE V.

FIG. 38. J. G. Young spermatid, centrosome divided. Nebenkern granules. Leitz Binoc. 4b $\times 1/12$ lens.

FIGS. 39, 40. Unstained. Young spermatid. Neutral red granules. Thread-like appearance in the nebenkern. Zeiss 6 oc. 2 mm. lens.

FIGS. 41, 42. J. G. Nebenkern dividing is now granular. Neutral red granules. Leitz 4b $\times 1/12$ lens.

FIG. 43. Unstained. Nebenkern divided. Zeiss 6 oc. 2 mm. lens.

FIG. 44. Unstained. Nebenkern sacs with the centrosome close to the nucleus. Neutral red granules. Zeiss 6 oc. 2 mm. lens.

FIG. 45. Unstained nebenkern sacs are slightly elongated. Zeiss 6 oc. 2 mm. lens.

FIG. 46. J. G. Nebenkern sacs elongated. Axial filament. Double middle piece body. Neutral red granules. Zeiss 6 oc. 2 mm. lens.

FIGS. 47, 48. J. G. The tail failed to develop but the mitochondria elongated and formed the double thread as usual, although the double thread is wound round and round within the cell. Leitz Binoc. 4b $\times 1/12$ lens.

FIG. 49. N. R. The cytoplasm of the tail is gathered into nodes and the neutral red granules appear. The mitochondria is now in the form of two homogenous long threads. Zeiss 6 oc. 2 mm. lens.

FIGS. 50, 51. J. G. Later stages of the spermatozoön.

